AD________________

Award Number: DAMD17-97-1-7151

TITLE: Regulation of Sialomucin Complex Expression and Its Effect on HER Receptor Interaction

PRINCIPAL INVESTIGATOR: Nebila Idris
Kermit Carraway, Ph.D.

CONTRACTING ORGANIZATION: University of Miami
Miami, Florida 33101

REPORT DATE: September 2001

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

20020904 046

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 2001	Final (15 Aug 97 - 14 Aug 01)

4. TITLE AND SUBTITLE
Regulation of Sialomucin Complex Expression and Its Effect on HER Receptor Interaction

5. FUNDING NUMBERS
DAMD17-97-1-7151

6. AUTHOR(S)
Nebila Idris
Kermit Carraway, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Miami
Miami, Florida 33101

E-Mail: nidris@hotmail.com

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT Sialomucin complex (SMC) is a heterodimeric glycoprotein complex consisting of a mucin subunit ASGP-1 and a transmembrane subunit ASGP-2, which is highly overexpressed on the surface of ascites 13762 rat mammary adenocarcinoma cells. ASGP-2 appears to be a ligand for the growth factor receptor ErbB2. In normal rat mammary gland the levels of both SMC and ErbB2 are sharply increased during pregnancy. In normal cultured mammary epithelial cells (MEC) SMC is post-transcriptionally regulated by Matrigel and TGFβ. SMC expression in the 13762 mammary adenocarcinoma cells is unaffected by Matrigel or TGFβ. In contrast, ErbB2 is maximally expressed when MEC are embedded in Matrigel. Although SMC and ErbB2 can be found in a complex in whole lactating rat mammary tissue, the two proteins are more readily co-immunoprecipitated from freshly isolated MEC, whose polarity is disrupted as well as in the tumor, which also has its polarity disrupted. Finally, in tumor cell lines that overexpress SMC, antibody binding to ErbB-2 is significantly reduced, while the overall levels of ErbB2 in the cells are not reduced. Thus, overexpression of SMC and formation of the SMC/ErbB2 complex may contribute to increased metastatic potential and decreased responsiveness of some breast cancers to anti-ErbB2 therapeutic agents.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer	27
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction

Sialomucin complex (SMC, rat Muc4) was originally discovered as the major glycoprotein complex on the surface of highly malignant, metastatic 13762 rat ascites mammary adenocarcinoma cells (Sherblom and Carraway, 1980). The complex consists of a peripheral, O-glycosylated mucin subunit ASGP-1 (Sherblom et al., 1980a, b), and an N-glycosylated integral membrane glycoprotein ASGP-2 to which ASGP-1 is tightly, but non-covalently, bound (Sherblom and Carraway, 1980; Hull et al., 1990). Recent studies have demonstrated that Muc4/SMC is the rat homolog of human MUC4 (Moniaux et al., 1999). Several studies suggest that the two-subunit Muc4/SMC is a multi-functional glycoprotein complex. Overexpression of Muc4/SMC can provide anti-recognition and anti-adhesive properties to tumor cells (Komatsu et al., 1997). Furthermore, Muc4/SMC expression in tumor cells reduces their killing by natural killer cells (Komatsu et al., 1999). ASGP-2 has two epidermal growth factor-like domains, which have all of the consensus residues present in active members of the epidermal growth factor family (Sheng et al., 1992). Moreover, Muc4/SMC has been shown to bind to and modulate phosphorylation of the receptor ErbB2 (Carraway et al., 1999). Thus, the transmembrane subunit ASGP-2 is proposed to modulate signaling through the epidermal growth factor family of receptors via its interaction with erbB2 (Carraway et al., 1999; Carraway et al., 1992). This interaction may play a role in the constitutive phosphorylation of erbB2 in the 13762 ascites cells (Juang et al., 1996) and the rapid growth of these cells in vivo. Sialomucin complex expression ahs been described in a number of normal secretory epithelial tissues in the adult rat including mammary gland (Rossi et al., 1996; McNeer et al., 1997) and appears to have multiple and complex regulatory mechanisms. Because overexpression of Muc4/SMC may lead o deleterious consequences, it is important to understand how expression of this protein is regulated as well as the consequences of its interaction with ErbB2. Thus, we are characterizing regulation of Muc4/SMC expression in normal mammary epithelial cells and 13762 mammary ascites tumor cells. Furthermore, we are characterizing the interactions between ASGP-2 and ErbB2 and the possible consequences of this interaction. For this final report we describe expression and interaction of Muc4/SMC and ErbB2 in normal mammary gland and tumor tissue. Muc4/SMC and ErbB2 form a complex in normal mammary tissue and mammary tumor cells. Overexpression of Muc4/SMC on tumor cells blocks antibody binding to ErbB2 that is dependent on the antibody isotype used. This complex formation may provide tumor cells a mechanism of Herceptin resistance. In the normal mammary gland, ErbB2 co-localizes with Muc4/SMC at the apical surfaces of the alveolar cells in lactating gland; however, another form of ErbB2, recognized by a different antibody, localizes to the basolateral surfaces of these cells. Moreover, ErbB2 phosphorylated on Tyr 1248 co-localizes with Muc4/SMC at the apical surface but not at the basolateral surfaces of these cells. These data indicate that Muc4/SMC and ErbB2 complex formation in the mammary gland is developmentally regulated and there are different forms of ErbB2 present in mammary epithelial cells that may have different functions in the mammary gland. Further, overexpression of Muc4/SMC on tumor cells may have both prognostic and therapeutic relevance.

This report summarizes the results of studies outlined in DAMD17-97-1-7151: Regulation of Sialomucin Complex Expression and Its Effect on HER Receptor Interaction. In these studies we have investigated the interaction of Muc4/SMC with the growth hormone receptor ErbB2 and its potential effects on cancer therapies. For a detailed discussion of regulatory mechanisms of Muc4/SMC in normal mammary epithelial cells and interactions of Muc4/SMC with ErbB2 in normal mammary gland and tumor cells (Tasks 1-5 in Statement of Work), please see the following appended manuscripts/reprints:

Price-Schiavi, S.A., Craway, C.A.C., Fregien, N.L., and carraway, K.L. (1998) Post-transcriptional regulation of a milk membrane protein, the Sialomucin complex, (Ascites sialoglycoprotein (ASGP)-1ASGP-2, Rat Muc4), by transforming growth factor β. J. Biol. Chem. 273, 35288-35237.

Price-Schiavi, S.A., Zhu, X., Aquinin, R., and Carraway, K.L. (200) Sialomucin Complex (Rat Muc4) is regulated by transforming growth factor β in mammary gland by a novel post-translational mechanism. J. Biol. Chem. 275, 17800-17807.

Zhu, X., Price-Schiavi, S.A., and Carraway, K.L. (2000) Extracellular regulated kinase (ERK)-dependent regulation of Sialomucin complex.Muc4 expression in mammary epithelial cells. Oncogene. 19, 4354-4361.

Price-Schiavi, S.A., Jepson, S., Li, P., Arango, M., Rudland, P.S., Yee, L., and Carraway, K.L. (2002) Rat Muc4 (Sialomucin complex) reduces binding of anti-ErbB2 antibodies to tumor cell surfaces, a potential mechanism for herceptin resistance. Int. J. Cancer. In press.

Price-Schiavi, S.A., Idris, N., Li, P., Carraway, C.A.C., and Carraway, K.L. Expression, location and interactions of ErbB2 and its intramembrane iigand Muc4 (Sialomucin Complex) in rat mammary gland during pregnancy. Manuscript in preparation.

Expression of Muc4/SMC in Human Breast Cancer

Cancer progression can be associated with aberrant expression of glycoproteins on tumor cell surfaces. Muc4/SMC is highly overexpressed on the surface of the highly malignant metastatic 13762 rat mammary adenocarcinoma with levels 100-fold higher than normal lactating mammary gland and 10,000-fold higher than normal rat mammary gland (Price-Schiavi et al., 1998; Rossi et al., 1996). By immunohistochemistry and immunoblot analysis we have shown that MUC4 is expressed in a minority of solid breast tumors and is overexpressed in the majority of more aggressive tumor cells from effusions of breast cancer patients (Komatsu et al., 1999). Moreover, it has been demonstrated that MUC4 is aberrantly expressed in a number of human malignancies (Walsh et al., 1993). To further investigate MUC4 expression in human breast cancer, we performed immunohistochemical staining of breast tumors from breast cancer patients. Paraffin-embedded infiltrating carcinoma specimens were tested for MUC4 expression by staining with anti-ASGP-1 monoclonal antibody 15H10. In these tumor specimens MUC4 stained throughout the ductal epithelium with more intense staining towards the lumenal surface (data not shown). Moreover, there was strong staining of cells invading the lumen of the duct and the surrounding tissue. About 30% of these tumor samples was strongly positive for MUC4 (Table I). As shown previously, breast cancer samples from breast cancer patient effusions showed an even higher level of positivity (Table I). To verify the staining of MUC4, we performed immunoblots on a strongly positive breast cancer sample compared to a negative sample (Fig. 1). As positive controls, we show samples from the 13762 ascites cells and Muc4/SMC-transfected A375 cells grown with or without tetracycline to turn Muc4/SMC OFF or ON, respectively (Fig. 1). Taken together with our previous data, these observations suggest a role for Muc4/SMC in human breast tumor progression (Komatsu et al., 1999).

Effect of antibody isotype on antibody binding to A375 Cells

Overexpression of Muc4/SMC blocks cell-cell and cell-matrix interactions by non-specific steric hindrance (Komatsu et al., 1999). Part of this demonstration was that when Muc4/SMC was overexpressed, cell adhesion to a number of different ECM components was inhibited, and the degree of inhibition was dependent on the expression level of Muc4/SMC and the number of mucin repeats the Muc4/SMC molecules contained. In the last report we demonstrated that overexpression of Muc4/SMC blocks antibody binding to ErbB2 on the surface of both A375 human melanoma and MCF-7 human mammary adenocarcinoma cell lines. In these studies we demonstrated that Muc4/SMC overexpression inhibits IgG1 isotype antibody binding to ErbB2 only, and we have previously reported that Muc4/SMC and ErbB2 can form a complex. Thus, the inhibition of anti-ErbB2 antibody binding by Muc4/SMC overexpression may be from steric hindrance due to the formation of the Muc4/SMC-ErbB2 complex. To test this idea, we measured cell surface antibody binding with a different, unrelated antibody in the presence or absence of Muc4/SMC expression. A375 cells were cultured for 72 hours in the presence or absence of tetracycline as described. Cells were harvested and stained with either anti-Fas IgG or anti-Fas IgM isotype antibodies. When stained with anti-Fas IgG antibodies, cell surface antibody binding is similar whether or not Muc4/SMC is expressed (Fig. 2A). However, when stained with larger, more bulky anti-Fas IgM antibodies, antibody binding is reduced by approximately 60% when Muc4/SMC is overexpressed (Fig. 2B). Although the differences in staining due to differences in the anti-Fas antibodies cannot be ruled out, these data suggest that inhibition of antibody binding is dependent on the class of antibody and suggest that inhibition of ErbB2 antibody binding may not be due entirely to nonspecific steric hindrance but instead from steric hindrance due to the formation of a Muc4/SMC-ErbB2 complex.

Co-immunoprecipitation of Muc4/SMC and ErbB-2 from A375 cells

We have previously demonstrated that Muc4/SMC and ErbB-2 can form a complex in co-infected insect cells, normal mammary epithelial cells, and 13762 mammary tumor cells. In our previous report we demonstrated that overexpression of Muc4/SMC blocks antibody binding to ErbB2 and that capping of Muc4/SMC with antibodies further reduces antibody binding to ErbB2 rather than increasing it. These data suggest that reduction of antibody binding may not be due to nonspecific steric hindrance but instead from steric hindrance from the specific formation of a Muc4/SMC/ErbB2 complex. To determine if there is some interaction between ErbB-2 and Muc4/SMC in the transfected A375 cells that may interfere with antibody binding to ErbB-2, a co-immunoprecipitation was performed. A375 cells expressing Muc4/SMC were lysed and immunoprecipitated with either anti-ASGP-2, anti-C-pep, or anti-ErbB-2 antibodies, and immunoprecipitates were subjected to immunoblot analysis with anti-ASGP-2 mAb 4F12. Muc4/SMC was readily detected in the anti-ErbB-2 immunoprecipitates but not in the non-immune rabbit serum control suggesting that Muc4/SMC and ErbB-2 form a complex in these cells (Fig. 3). These data suggest an interaction between Muc4/SMC and ErbB-2 that may interfere with antibody binding to ErbB-2.

Effect of Muc4/SMC overexpression on antibody binding to ErbB2 on human breast cancer cells

The A375 cells are human melanoma cells, not breast cancer cells, and Herceptin is approved as a treatment for metastatic breast cancer. Thus, to determine if overexpression of Muc4/SMC blocks ErbB2 antibody binding on breast tumor cells, MCF-7 cells stably transfected with tetracycline regulatable Muc4/SMC were analyzed in a manner similar to that described for the A375 cells described in the last report. To determine what effect Muc4/SMC overexpression has on Herceptin binding to ErbB2 on the surface of breast cancer cells, MCF-7 cells were cultured in the presence or absence of tetracycline for 72 hours. Cells were harvested in enzyme-free cell dissociation buffer and analyzed by flow cytometry with Herceptin (at a 100, 10, or 1 μg/ml dilution). As with the A375 cells, there was reduced Herceptin binding when MCF-7 cells expressed high levels of Muc4/SMC. However, unlike the A375 cells, MCF-7 cells expressing high levels of Muc4/SMC showed a 25-40% reduction in Herceptin binding compared to MCF-7 cells not expressing Muc4/SMC, regardless of the concentration of Herceptin used for staining (Fig. 4). These results suggest that for breast cancer cells, overexpression of Muc4/SMC may provide a block to antibody-based therapies even at the lower therapeutic doses.

Localization of Muc4/SMC and ErbB2 in normal developing mammary tissue

We have previously demonstrated by immunoblot and immunohistochemical analysis that Muc4/SMC is developmentally regulated and is localized on the lumenal surfaces of ductal and alveolar epithelial cells in the normal developing rat mammary gland (Rossi et al., 1996; Price-Schiavi et al, 1998; Li *et al.*, 2001). Further, we have shown that different anti-ErbB2 antibodies recognize different forms of ErbB2 and show differential staining within the same tissue (Idris et al., 2001). To compare cellular localization of Muc4/SMC and ErbB2 in lactating mammary gland and to compare ErbB2 staining pattern with different anti-ErbB2 antibodies, whole mammary tissue isolated from lactating rats was analyzed by immunohistochemical staining with antibodies directed against Muc4/SMC (ASGP-2) and ErbB2. As expected, Muc4/SMC was stained on the apical surfaces of ductal and alveolar epithelial cells. When stained with Neomarkers anti-ErbB2 antibody 1, a polyclonal antibody directed against a peptide in the C-terminal region of ErbB2, ErbB2 was detected at the apical surfaces of the alveolar epithelial cells (Fig. 5). However, when stained with Dako anti-ErbB2 (Herceptest), ErbB2 staining was localized largely on the basolateral surfaces of the alveolar epithelial cells. No staining was detected with any of the antibodies in the myoepithelial or stromal cells. Thus, as shown in female reproductive tissues, different anti-ErbB2 antibodies stain ErbB2 differently in lactating mammary tissue, suggesting that these antibodies recognize different forms of ErbB2. Moreover, these data suggest that one form of ErbB2 co-localizes with Muc4/SMC at the apical surfaces of the secretory mammary epithelium.

The differential localization of ErbB2 in lactating mammary gland raises a question about the nature of the two forms of this protein. We have recently demonstrated that complex formation between Muc4/SMC and ErbB2 leads to phosphorylation of ErbB2 Tyr1248. Further, we have demonstrated in female reproductive tract tissues that different forms of ErbB2 are differentially localized depending on the antibody used for staining and that this difference is correlated with its phosphorylation state. To determine if the differential localization of ErbB2 in the lactating mammary gland is also correlated with its phosphorylation state, we stained lactating mammary tissue with Neomarkers anti-ErbB2 antibody 17 and 18. NeoMarkers anti-ErbB2 antibody 18 recognizes a phosphorylated peptide in the C-terminal region of ErbB2, while antibody 17 recognizes only the unphosphorylated form of this peptide. When lactating mammary tissue was stained with the anti-phospho-ErbB2 antibody, staining was localized to the apical surface of the alveolar epithelial cells (Fig. 6). Likewise, when stained with antibody 17, ErbB2 staining was also localized to the apical surface of the alveolar epithelial cells. There was little ErbB2 detected with either antibody at the basolateral surfaces of the cells as seen with the Dako anti-ErbB2 antibody. Taken together, these data suggest that both Tyr 1248 phosphorylated and Tyr 1248 unphosphorylated forms of ErbB2 co-localize with Muc4/SMC at the apical surface of the mammary alveolar epithelium. Moreover, the form of ErbB2 recognized by the Dako antibody at the basolateral surface of the alveolar epithelium, which is not co-localized with Muc4/SMC, is not phosphorylated on Tyr 1248.

Key research accomplishments to date:

1. Muc4/SMC is developmentally regulated in normal rat mammary gland largely by a post-transcriptional mechanism.

2. Matrigel (reconstituted ECM) post-transcriptionally regulates SMC levels in normal rat MEC by inhibition of Muc4/SMC precursor synthesis.

3. Muc4/SMC levels in 13762 MAT-B1 tumor cells are unaffected y Matrigel.

4. Muc4/SMC is post-translationally regulated in normal rat MEC by TGFβ by disruption of Muc4/SMC precursor processing. (note that this is a different mechanisms than that described for Matrigel.)

5. Muc4/SMC expression is unaffected by TGFβ in 13762 MAT-B1 tumor cells.

6. Muc4/SMC and ErbB2 have similar expression patterns in normal developing rat mammary gland.

7. Muc4/SMC and ErbB2 can form a complex in both virgin and lactating mammary gland.

8. Muc4/SMC and ErbB2 have different mechanisms of regulation in culture normal rat mammary epithelial cells.

9. The inhibitory effect of TGFβ on Muc4/SMC expression can be blocked by IFNγ in a time and dose dependent manner.

10. Overexpression of Muc4/SMC on tumor cell surfaces can block antibody binding (including Herceptin) to ErbB2 by steric hindrance from complex formation with ErbB2.

11. There are different forms of ErbB2 in normal mammary gland that stain differently with different anti-ErbB2 antibodies that may have different functions this tissue.

Reportable outcomes:

1. Papers/manuscripts:

Price-Schiavi, S. A., Carraway, C. A. C., Fregien, N. L., and Carraway, K. L. (1998) Post-transcriptional regulation of a milk membrane protein, the sialomucin complex, (Ascites sialoglycoprotein (ASGP)-1/ASGP-2, Rat Muc4), by transforming growth factorβ. J. Biol. Chem. 273, 35288 – 35237

Carraway, K.L., Price-Schiavi, S.A., Zhu, X., and Komatsu, M. (1999) Regulation of expression of sialomucin complex (rat Muc4), the intramembrane ligand for ErbB2, at the transcriptional, translational and post-translational levels in rat mammary gland. Cancer Control 6, 613-614

Carraway, K.L., Price-Schiavi, S.A., Komatsu, M., Idris, N., perez, A., Li, P., Jepson, S., Zhu, X., Carvajal, M.E., and Carraway, C.A.C. (2000) Multiple facets of sialomucin complex/MUC4, a membrane mucin and ErbB-2 ligand, in tumors and tissues (Y2K update) Frontiers in Bioscience 5, 95-107

Price-Schiavi, S. A., Zhu, X., Aquinin, R., and Carraway, K. L. (2000) Sialomucin Complex (Rat Muc4) is regulated by transforming growth factor β in mammary gland by a novel post-translational mechanism. J Biol Chem. 275, 17800-7

Zhu, X., Price-Schiavi, S. A., and Carraway, K. L. (2000) Extracellular regulated kinase (ERK)-dependent regulation of sialomucin complex/Muc4 expression in mammary epithelial cells. Oncogene. Sep 7;19(38):4354-4361

Price-Schiavi, S. A., Jepson, S., Li, P., Carvajal, M. E., Komatsu, M., and Carraway, K. L. (2002) Rat Muc4 (Sialomucin complex) reduces binding of anti-ErbB2 antibodies to tumor cells surfaces, a potential mechanism for Herceptin resistance. Int. J. Cancer. In press.

Price-Schiavi, S. A., Idris, N., Li, P., Carraway, C. A. C., and Carraway, K. L. Interaction of sialomucin complex (SMC, rat Muc4) with ErbB-2 in developing rat mammary gland and 13762 mammary tumor cells. Manuscript under revision.

Price-Schiavi, S. A., Zhu, X., Falkenburg, R. V., Ramsauer, V., and Carraway, K. L. Interferon gamma (IFN-γ) blocks downregulation of sialomucin complex (SMC/Rat Muc4) expression in normal rat mammary epithelial cells. Manuscript in preparation

2. Abstracts and presentations:

Post-transcriptional regulation of a milk membrane protein, Sialomucin complex, by TGFβ
Price-Schiavi, S.A., Carraway, C.A.C., Fregien, N.L., and Carraway, K.L.
Poster presentation at American Society for Cell Biology, San Francisco CA, December, 1998

Post-transcriptional regulation of sialomucin complex in normal rat mammary gland by TGFβ
Price-Schiavi, S.A., Fregien, N.L., Carraway, C.A.C., and Carraway, K.L.
Poster presentation at Nature BioTechnology Winter Symposium, Miami FL, February, 1999

Characterization of the TGFβ effect on sialomucin complex (Rat MUC-4) expression in normal rat mammary epithelial cells
Price-Schiavi, S.A., Zhu, X., and Carraway, K.L.
Poster presentation at American Society for Biochemistry and Molecular Biology, San Francisco CA, May 1999

Mechanisms for post-transcriptional regulation of SMC expression in normal rat mammary epithelial cells
Price-Schiavi, S.A., Aquinin, R., and Carraway, K.L.
Poster presentation at Gordon Conference on Mammary Gland Biology, Henniker NH June 1999

Regulation of sialomucin complex in normal rat mammary gland
Oral presentation at Nature BioTechnology Winter Symposium, Miami FL, February, 2000

Rat Muc4 (Sialomucin complex) reduces binding of anti-ErbB2 antibodies to tumor cells surfaces, a potential mechanism for Herceptin resistance
Price-Schiavi, S. A., Jepson, S., Li, P., Carvajal, M. E., Komatsu, M., and Carraway, K. L.
Poster presentation at Gordon Conference on Mammary Gland Biology, Italy, 2000

Interferon gamma (IFN-γ) blocks downregulation of sialomucin complex (SMC/Rat Muc4) expression in normal rat mammary epithelial cells
Price-Schiavi, S.A., Zhu, X., Falkenburg, R.V., Ramsauer, V., and Carraway, K. L.
Poster presentation at AACR meeting, New Orleans, LA, 2001

3. Degrees obtained that were supported by award DAMD17-97-1-7151:

Shari A. Price-Schiavi, Doctor of Philosophy in Molecular Cell and Developmental Biology, University of Miami, December 1999

4. Employment received on experiences/training supported by DAMD17-97-1-7151:

Shari A. Price-Schiavi, Post-doctoral associate, Department of Cell Biology and Anatomy, University of Miami, laboratory of Dr. Kermit Carraway, January – June 2000

Shari A. Price-Schiavi, Post-doctoral associate, Sylvester Comprehensive Cancer Center University of Miami, laboratory of Kelvin Lee, M.D., June 2000 – December 2000

Shari A. Price-Schiavi, Scientist, Sunol Molecular Corporation, Miramar, FL, January 2001-present

Conclusion

The overall goal of these studies was to elucidate the mechanisms of regulation of Muc4/SMC in normal mammary epithelia and to determine what effects Muc4/SMC complex formation with ErbB2 would have on cells. ErbB2 and Muc4/SMC have both been implicated in cancer and normal functions such as development and protection (Alroy and Yarden, 1997; Carraway *et al.*, 2000). Thus, it is important to understand what contribution each of these proteins and their complex has on development of the normal mammary gland and tumor progression. Mucr4/SMC is developmentally regulated n normal rat mammary gland by a post-transcriptional mechanism. Although a number of factors have some effect on Muc4/SMC levels in primary mammary epithelial cell cultures, Matrigel and TGFβ can mimic the post-transcriptional regulation of Muc4/SMC seen in vivo. Interestingly, the regulatory mechanisms for these two factors are different. Matrigel inhibits synthesis of the Muc4/SMC precursor, while TGFγβ interferes with Muc4/SMC precursor processing. These data indicate both post-transcriptional and post-translational mechanisms of regulation, which further illustrates the complexity of regulating mammary gene expression.

Muc4/SMC and ErbB2 have different expression patterns and regulatory mechanisms. However, they are both expressed at maximal levels in late pregnant and lactating mammary gland and may form a complex at these stages of mammary development. Incidentally, expression of neuregulin, another ErbB2 ligand, is maximal during late pregnancy and lactation and has been reported to be expressed in the mammary stroma (Yang *et al.*, 1995). Indeed, Muc4/SMC and ErbB2 could be co-immunoprecipitated as a complex from lactating mammary tissue but not from virgin mammary tissue. Because the anti-C-pep antibody used for co-immunoprecipitations recognizes only membrane bound Muc4/SMC, the Muc4/SMC complexed with ErbB2 in mammary gland appears to be membrane-associated. Because the two proteins were also co-immunoprecipitated from isolated mammary epithelial cells, the Muc4/SMC complex must be present in to the epithelial cells. Moreover, consistent with our previous studies, the complex does not appear to involve ErbB3 (Carraway *et al.*, 1999b). These data suggest that Muc4/SMC-ErbB2 complex formation is a normal physiological phenomenon and is developmentally regulated in the normal developing rat mammary gland.

In the lactating mammary gland Muc4/SMC and a portion of ErbB2 are co-localized. Muc4/SMC is expressed at the apical surfaces of the alveolar epithelial cells in the lactating mammary gland. On the other hand, ErbB2 is expressed on both the apical and basolateral surfaces of the alveolar mammary epithelial cells, with each form being recognized by different anti-ErbB2 antibodies, suggesting that there are different forms of ErbB2 present at the apical and basolateral surfaces of mammary alveolar cells. Similarly, in the rat oviduct the NeoMarkers antibody 1 detected ErbB2 at the apical surfaces of the epithelial cells, while the Dako antibody recognized ErbB2 at the lateral surfaces (Idris and Carraway, 2001). In support of this idea, we investigated the phosphorylation states of ErbB2 in the mammary gland. Staining for phosphorylated ErbB2 reveals that both Tyr 1248-phosphorylated as well as Tyr 1248-unphosphorylated ErbB2 co-localizes with Muc4/SMC at the apical surface of the alveolar epithelial cells. ErbB2 localized at the basolateral surfaces of these cells was not stained with the phospho-ErbB2 antibody or the antibody recognizing the unphosphorylated form. This lack of recognition could be due to lack of phosphorylation of this peptide or block of these epitopes by other signaling components bound to the basolateral form of ErbB2. Taken together these data indicate that there are different forms of ErbB2 at the apical and basolateral surfaces of the alveolar mammary epithelial cells.

Differential recognition of ErbB2 forms by different antibodies has been reported (DiGiovanna, 1997; Darcy *et al.*, 2000). This notion is confirmed by numerous reports of ErbB2 localization in mammary gland. Darcy *et al.* reported that ErbB2 was present at high levels in virgin, pregnant, and involuting mouse mammary gland but not in lactating mammary gland. Moreover, they report that ErbB2 is localized to all cell types of the mammary gland at various stages, including stromal cells (Darcy *et al.*, 2000). On the other hand Schroeder and Lee report that ErbB2 is localized to all cell types in immature mammary gland but restricted to epithelia in the differentiated gland (Schroeder and Lee, 1998). Press *et al.* localized ErbB2 to the basoateral but not the apical cell membranes of ductal and lobular epithelium, while Darcy et al. describe localization to both apical and basolateral surfaces (Press *et al.*, 1990, Darcy *et al.*, 2000). Our data indicate that ErbB2 is present at high levels in the lactating rat mammary gland and that, depending on which antibody is used, it can be localized to both basolateral and apical surfaces of the alveolar epithelial cells. Taken together, these data indicate that different ErbB2 antibodies localize ErbB2 differently in developing mammary tissue although one cannot rule out differences in the detection method, assay conditions, species and reproductive history of donor for the contradictory ErbB2 expression and localization.

In addition to suggesting a role in maintenance of the lactating mammary gland, our studies raise a note of caution about the use of ErbB2 antibody staining in determining the prognosis and course of treatment for breast cancer patients. We have demonstrated that overexpression of Muc4/SMC can block antibody binding to ErbB2, and these studies indicate that co-expression and complex formation with Muc4/SMC may cause differential antibody recognition to ErbB2 in histological preparations (Price-Schiavi *et al.*, submitted). Thus, it may be clinically relevant to determine if specific forms of ErbB2 and co-expression and complex formation with Muc4/SMC influence responses to treatment and correlate with clinical outcome.

Proper localization of receptors in polarized cells is critical to their normal function. Our data indicate differential localization of ErbB2 in the mammary gland, raising a question about the mechanism for localization. Borg *et al.* report that ERBIN, a PDZ domain-containing protein, can maintain ErbB2 at the basolateral surfaces of polarized epithelial cells through interactions in the C-terminal domains of ERBIN and ErbB2 and that ERBIN loss of function mutations result in mislocalization of ErbB2 (Borg *et al.*, 2000). Similarly, we have shown that Muc4/SMC can form a complex with ErbB2 and potentiate phosphorylation of this receptor (Carraway *et al.*, 1999b). Through its interaction with ErbB2, which appears to form during its intracellular transit, Muc4/SMC may redirect a portion of ErbB2 to the apical surface of the cells, while ERBIN or other PDZ domain-containing proteins may direct another portion to the basolateral surface (Fig. 7). In support of this idea, we show that Tyr 1248 phosphorylated ErbB2 co-localizes with Muc4/SMC at the apical surface of the alveolar cells and not at the basolateral surface. Thus, different interactions of ErbB2 with proteins such as Muc4/SMC and ERBIN may allow for polarized expression and function of ErbB2. Further, differential antibody staining can be explained by masking of epitopes by other signaling molecules recruited to the different sites of ErbB2 interaction.

Four types of observations suggest that the Muc4/SMC-ErbB2 complexes may be involved in cellular signaling. 1) ErbB2 immunoprecipitated from plasma membranes of the 13762 ascites cells as a complex with Muc4/SMC has a highly active tyrosine kinase (Juang *et al.*, 1996). Moreover, this complex is associated with elements of downstream signaling pathways, including components of the Shc-Ras-MAPK mitogenic cascade (Carraway *et al.*, 1999a). 2) Receptor phosphorylation is increased in insect cells expressing ErbB2 plus ASGP-2, but not those expressing ErbB2 alone or the other three receptors plus ASGP-2 (unpublished observations). 3) ErbB2 immunoprecipitated from A375 cells expressing Muc4/SMC under control of a tetracycline-inducible promoter was more heavily phosphorylated than that from the Muc4/SMC negative cells, indicating that Muc4/SMC (ASGP-2) can activate phosphorylation of ErbB2, presumably by an autophosphorylation mechanism. Furthermore, when these cells were treated with the ErbB3 ligand neuregulin, which activates the ErbB2/ErbB3 complex, ErbB2 phosphorylation was increased to a much greater extent in the presence of Muc4/SMC than in its absence, showing that Muc4/SMC can potentiate the effects of the neuregulin. Moreover, phosphorylation of ErbB3 was also potentiated. 4) Expression of Muc4/SMC in stably transfected A375 cells leads to phosphorylation of Tyr 1248, which has been implicated in neoplasmic transformation (Eppenberger-Castori *et al.*, 2001; Thor *et al.*, 2000). Based on these combined results, we have proposed that Muc4/SMC can act as an intramembrane, intracrine modulator of ErbB2, and could participate in epithelial or tumor cell regulation, in cells in which it is expressed.

An important finding in the course of these studies was the demonstration that overexpression of Muc4/SMC on the surface of A375 human melanoma and MCF-7 human breast carcinoma cells inhibits binding of several different anti-ErbB2 antibodies, including Herceptin. Overexpression of Muc4/SMC does not affect the expression levels of ErbB2 in either cell line. Furthermore, binding of other, unrelated antibodies was dependent on the isotype used, and capping of Muc4/SMC caused a greater inhibition of anti-ErbB2 antibody binding than when Muc4/SMC was not capped. These data suggest that the inhibition of anti-ErbB2 antibody binding is due to steric hindrance from the formation of a Muc4/SMC-ErbB2 complex.

Overexpression of Muc4/SMC on the surfaces of tumor cells disrupts cell-cell and cell-matrix interactions and provides protection from immune surveillance (Komatsu et al., 1999; Komatsu et al., 1997; Komatsu et al., 2000). The anti-adhesive and protective functions of Muc4/SMC have been attributed to the rigid, extended structure of the highly O-glycosylated mucin subunit, as the degree of the anti-adhesive and protective effects are dependent on the size of the Muc4/SMC molecule (i.e. the number of highly O-glycosylated tandem repeats the molecule contains) and the level of cell surface expression (Komatsu et al., 1999; Komatsu et al., 1997). On the basis of electron microscopic studies of other mucins, we have estimated that the Muc4/SMC molecule extends approximately 500 nm above the cell surface (Komatsu et al., 1997; Jentoft, 1990; Wesseling et al., 1996; Cyster et al., 1991). Other cell surface molecules including adhesion molecules like integrins have been estimated to be approximately 30 nm long Codington and Fim, 1983; Becker et al., 1989). The high degree of O-glycosylation on the ASGP-1 subunit not only contributes to the extended structure of Muc4/SMC but also gives it considerable bulk. Thus, overexpression of Muc4/SMC masks the entire cell surface and blocks cell-cell and cell-matrix interactions that involve several different cell surface molecules. This nonspecific steric hindrance interferes with cellular functions elicited by these cell-cell and cell-matrix interactions. Given that Muc4/SMC overexpression can sterically block cell surface molecules such as adhesion and MHC molecules, this non-specific steric hindrance may also contribute to blocking of antibody binding to other cell surface proteins such as ErbB2. Importantly, human MUC4 is substantially larger than the rat homolog Muc4/SMC. In the mucin subunit, Muc4/SMC has twelve tandem repeats of 125 amino acids each, while human MUC4 only has three of these tandem repeats. However, the human homolog contains an unrelated tandem repeat sequence of sixteen amino acids each, which, due to genetic polymorphism, may be repeated 140 to 400 times. This additional repeat domain allows the human MUC4 to be two to three times as large as Muc4/SMC. Thus, it would be expected that human MUC4 would mask the cell surface much more efficiently than rat Muc4/SMC and cause a more pronounced anti-recognition effect.

It has been established that a significant number of ErbB2-expressing breast tumors are not responsive to Herceptin (Baselga et al., 1996; Pegram et al., 1998; Cobleigh et al., 1999). Several models for Herceptin (4D5) resistance have been put forward. One group suggested that intracellular expression of the extracellular domain of ErbB2 interferes with internalized ErbB2/4D5 complexes (Scott et al., 1993). Others propose that since there is a correlation between ErbB-3 expression and 4D5 sensitivity in some human tumor cells, strong proliferative signals generated from an ErbB2/ErbB-3 complex may lead to growth dependency during tumor development (Lewis et al., 1993; Lewis et al., 1996). Lane et al. suggest that Herceptin resistant tumor cells utilize alternative signaling pathways to override ErbB2 receptor inhibition (Lane et al., 2000). We propose another model whereby expression of Muc4/SMC on tumor cells and formation of a complex with ErbB2 provides a specific steric block to anti-ErbB2 antibody (Herceptin) binding. The reduced binding of antibody would lead to a reduction in cytostatic effects and sensitization to other chemotherapies. Moreover, signaling from the Muc4/SMC/ErbB2 complex would lead to further proliferation and progression of the tumor.

What could be the function of a Muc4/SMC-ErbB2 complex in lactating mammary gland? We have shown that Muc4/SMC can act as an anti-apoptotic agent, possibly through complex formation and signaling from ErbB2. (Komatsu *et al.*, 2001). Overexpression of ErbB2 in mammary tumors is associated with enhanced survival (Kumar *et al.*, 1996; Lazar *et al.*, 2000). Further, mammary specific overexpression of ErbB2 or Muc4/SMC leads to hyperplasia (unpublished observations), consistent with the idea that Muc4/SMC can repress apoptosis. Thus, we can propose the following model. Muc4/SMC levels are repressed in virgin mammary gland when cell turnover is relatively high by extracellular matrix and TGF-β (Price-Schiavi *et al.*, 1998a, 2000). During pregnancy changes in the extracellular matrix and decreased active TGF-β allow for higher expression of Muc4/SMC. Changes occurring during pregnancy also increase levels of ErbB2. During late pregnancy and lactation Muc4/SMC and ErbB2 form a complex and allow for survival and maintenance of the mammary gland acinar epithelial cells. During involution, the severe downregulation of both ErbB2 and Muc4/SMC contribute to the massive apoptosis and reorganization of the mammary gland. In the case of tumor cells, overexpression of ErbB2 can lead to loss of TGF-β responsiveness. This, in turn, can lead to upregulation of Muc4/SMC, which when overexpressed, leads to depolarization of the tumor cell. This depolarization allows for more Muc4/SMC to complex with ErbB2 to contribute to tumor progression and its deleterious consequences.

It is now common to screen breast tumors for a variety of molecular markers such as estrogen receptor and ErbB2 to determine the best course of treatment for a breast cancer patient. Screening for another membrane mucin, MUC1, which correlates with poor clinical prognosis, is now being performed (Duffy et al., 2000; McGuckin et al., 1995). There is now increasing evidence that MUC4 is aberrantly expressed in a number of different cancers including breast cancers. What correlation MUC4 expression or MUC4/ErbB2 complex formation may have with clinical prognosis is as yet unknown. However, MUC4 expression provides a steric repression to anti-ErbB2 antibody binding. Thus, along with other tumor markers, it may be useful to screen for MUC4 expression in determining the best course of treatment for a breast cancer patient.

References

Alroy I, and Yarden Y. (1997). *FEBS Let.* **410,** 83-86

Baselga J, Tripathy D, Mendelsohn J, Baughman S, Benz CC, Dnatis L, Henderson IC, Norton L (1996) *J. Clin. Oncol.* **14**, 737-744

Becker JW, Erickson HP, Hoffman S, Cunningham BA, and Edelman GM. (1989) *Proc. Natl. Acad. Sci. USA*. **86**, 1088-1092

Borg JP, Marchetto S, Le Bivic A, Ollendorff V, Jaulin-Bastard F, Saito H, Fournier E, Adelaide J, Margolis B, Birnbaum D. (2000). *Nature Cell Biol.* **2**, 407-414

Carraway KL III, Rossi EA, Komatsu M, Price-Schiavi SA, Huang D, Guy PM, Carvajal ME, Fregien N, Carraway CAC, and Carraway KL. (1999). *J. Biol. Chem.* **274**, 5263-5266

Carraway KL, Fregien N, Carraway CAC, and Carraway KL III (1992) *J. Cell Sci.* **103**, 299-307

Carraway KL, Price-Schiavi SA, Komatsu M. Idris N, Perez A, Li P, Jepson S, Zhu X, Carvajal ME, and Carraway CAC. (2000). *Front. Bioscience* **5**, D95-D107

Carraway, CAC, Carvajal ME, and Carraway KL. (1999a). *J. Biol. Chem.* **274**, 25659-25667

Cobleigh MA, Vogel CL, Tripathy D, Robert NJ, Scholl S, Fehrenbacher L, Wolter J, Paton V, Shak S, Lieberman G, and Slamon DJ. (1999) *J. Clin. Oncol.* **17**, 2639-2648

Codington JF, and Fim DM. (1983) *Biomembranes* **11**, 207-258

Cyster JG, Shotton DM, and Williams AF. (1991) *EMBO J.***10**, 893-902

Darcy KM, Zangani D, Wohlhueter AL, Huang RY, Vaughan MM, Russell JA, Ip MM (2000) *J. Histochem Cytochem* **48**, 63-80

DiGiovanna MP. (1997). *Analitical Biochem.* **247**, 167-70

Duffy MJ, Shering S, Shery F, McDermott E, O'Higgins N. (2000) *Int. J. Biol. Markers.* **15**, 330-333

Eppenberger-Castori S, Kueng W, Benz C, Caduff R, Varga Z, Bannwart F, Fink D, Dieterich H, Hohl M, Muller H, Paris K, Schoumacher F, Eppenberger U. (2001). *J. Clin. Oncol.* **19**, 645-656

Hull, SR, Sheng Z, Vanderpuye OA, David C, and Carraway KL. (1990) *Biochem. J.* **265**, 121-129

Idris N, Carraway CAC, and Carraway KL. (2001) *J Cell Physiol.* **189**, 162-70

Jentoft N (1990) *Trends Biochem. Sci.* **15**, 291-294
Juang SH, Carvajal ME, Whitney M, Liu Y, and Carraway CAC. (1996). *Oncogene,* **12**, 1033-1042

Komatsu M, Carraway CAC, Fregien NL, and Carraway KL. (1997) *J. Biol. Chem.* **272**, 33245-33254

Komatsu M, Jepson S, Arango ME, Carraway CAC, and Carraway KL. (2001). **20**, 461-470

Komatsu M, Tatum L, Altman NH, Carraway CAC, and Carraway KL. (2000). *Int J Cancer.* **87**, 480-6

Komatsu M, Yee L, and Carraway KL. (1999) *Cancer Res.* **59**, 2229-2236

Kumar R, Mandal M, Lipton A, Harvey H, and Thompson CB. (1996). *Clinical Cancer Research* **2**, 1215-1219

Lane HA, Beuvink I, Motoyama AB, Daly JM, Neve RM, Hynes NE. (2000) *Mol. Cell. Biol.* **20**, 3210-3223

Lazar H, Baltzer A, Gimmi C, Marti A, Jaggi R. (2000). *Int. J. Cancer* **85**, 578-583

Lewis GD, Figari I, Fendly B, Wong WL, Carter P, Gorman C, Shepard HM. (1993) *Cancer Immunol. Immunother.* **37**, 255-263

Lewis GD, Lofgren JA, McMurtrey AE, Huijens A, Fendly BM, Bauer KD, and Sliwkowski MX. (1996) *Cancer Res.* **56**, 1457-1465

Li P, Price-Schiavi SA, Rudland PS, and Carraway KL. (2001). *J. Cell. Physiol.* **186**, 397-405

McGuckin MA, Walsh MD, Hohn BG, Ward BG, Wright RG. (1995) *Hum. Pathol.* **26**, 432-439

McNeer RR, Price-Schiavi SA, Komatsu M, Fregien N, Carraway CAC, and Carraway KL. (1997). *Front. Biosci.* **2**, 449-459

Moniaux N, Nollet S, Porchet N, Degand P, Laine A, and Aubert J-P. 1999) *Biochem. J.* **338**, 325-333

Pegram MD, Lipton A, Hayes DF, Weber BL, Baselga JM, Tripathy D, BalyD, Baughman SA, Twaddell T, Glaspy JA, Slamon DJ. (1998) *J. Clin. Oncol.* **16,** 2659-2671

Press F., Cordon-Cardo C, Slamon DJ. (1990). *Oncogene*, **5**, 953-962

Price-Schiavi SA, Carraway CAC, Fregien N, and Carraway KL. (1998a). *J. Biol. Chem.* **273**, 35228-35237

Price-Schiavi SA, Zhu X, Aquinin R, and Carraway KL. (2000). *J. Biol. Chem.* **275**, 17800-17807

Rossi EA, McNeer R, Price-Schiavi SA, Komatsu M, Van den Brande JMH, Thompson JF, Carraway CAC, Fregien NL, and Carraway KL. (1996). *J. Biol. Chem.* **271**, 33476-33485

Schroeder JA and Lee DC. (1998). *Cell Growth Differ.* **9**, 451-464

Scott GK, Robles R, Park JW, Montgomery PA, Daniel J, Holmes WE, Lee J, Keller GA, Li-WL, Fendly BM, Wood WI, Shepard HM, and Banz CC. (1993) *Mol. Cell. Biol.* **13**, 2247-2257

Sheng Z, Wu K, Carraway KL, and Fregien N. (1992) *J. Biol. Chem.* **267**, 16341-16346

Sherblom AP and Carraway KL. (1980). *J. Biol. Chem.* **255**, 12051-12059

Sherblom, AP, Buck, RL, and carraway, KL (1980) *J. Biol. Chem.* **255**, 783-790

Sherblom, AP, Huggins, JW, Chesnut, RW, Buck RL, Ownby CL, Dermer GB, and Carraway KL (1980) *Exp. Cell Res.* **126**, 417

Thor AD, Liu S, Edgerton S, More D 2nd, Kasowitz KM, Benz CC, Stern DF, DiGiovanna MP. (2000). *J. Clin. Oncol.* **18**, 3230-3239

Walsh MD, McGuckin MA, Devine PL, Hohn BG, and Wright RG. (1993) *J. Clin. Pathol.* **46**, 922-925

Wesseling J, van der Valk SW, and Hilkens J. (1996) *Mol. Biol. Cell.* **7**, 565-577

Yang Y, Spitzer E, Meyer D, Sachs M, Niemann C, Hartmann G, Weidner KM, Birchmeier C, and Birchmeier W. (1995). *J Cell Biol.* **131**, 215-26

Bibliography of publication supported by DAMD17-97-1-7151:

Price-Schiavi, S. A., Carraway, C. A. C., Fregien, N. L., and Carraway, K. L. (1998) Post-transcriptional regulation of a milk membrane protein, the sialomucin complex, (Ascites sialoglycoprotein (ASGP)-1/ASGP-2, Rat Muc4), by transforming growth factorβ. J. Biol. Chem. 273, 35288 – 35237

Carraway, K.L., Price-Schiavi, S.A., Zhu, X., and Komatsu, M. (1999) Regulation of expression of sialomucin complex (rat Muc4), the intramembrane ligand for ErbB2, at the transcriptional, translational and post-translational levels in rat mammary gland. Cancer Control 6, 613-614

Carraway, K.L., Price-Schiavi, S.A., Komatsu, M., Idris, N., perez, A., Li, P., Jepson, S., Zhu, X., Carvajal, M.E., and Carraway, C.A.C. (2000) Multiple facets of sialomucin complex/MUC4, a membrane mucin and ErbB-2 ligand, in tumors and tissues (Y2K update) Frontiers in Bioscience 5, 95-107

Price-Schiavi, S. A., Zhu, X., Aquinin, R., and Carraway, K. L. (2000) Sialomucin Complex (Rat Muc4) is regulated by transforming growth factor β in mammary gland by a novel post-translational mechanism. J Biol Chem. 275, 17800-7

Zhu, X., Price-Schiavi, S. A., and Carraway, K. L. (2000) Extracellular regulated kinase (ERK)-dependent regulation of sialomucin complex/Muc4 expression in mammary epithelial cells. Oncogene. Sep 7;19(38):4354-4361

Price-Schiavi, S. A., Jepson, S., Li, P., Carvajal, M. E., Komatsu, M., and Carraway, K. L. (2002) Rat Muc4 (Sialomucin complex) reduces binding of anti-ErbB2 antibodies to tumor cells surfaces, a potential mechanism for Herceptin resistance. Int. J. Cancer. In press.

Bibliography of meeting abstracts:

Post-transcriptional regulation of a milk membrane protein, Sialomucin complex, by TGFβ
Price-Schiavi, S.A., Carraway, C.A.C., Fregien, N.L., and Carraway, K.L.
Poster presentation at American Society for Cell Biology, San Francisco CA, December, 1998

Post-transcriptional regulation of sialomucin complex in normal rat mammary gland by TGFβ
Price-Schiavi, S.A., Fregien, N.L., Carraway, C.A.C., and Carraway, K.L.
Poster presentation at Nature BioTechnology Winter Symposium, Miami FL, February, 1999

Characterization of the TGFβ effect on sialomucin complex (Rat MUC-4) expression in normal rat mammary epithelial cells
Price-Schiavi, S.A., Zhu, X., and Carraway, K.L.
Poster presentation at American Society for Biochemistry and Molecular Biology, San Francisco CA, May 1999

Mechanisms for post-transcriptional regulation of SMC expression in normal rat mammary epithelial cells
Price-Schiavi, S.A., Aquinin, R., and Carraway, K.L.
Poster presentation at Gordon Conference on Mammary Gland Biology, Henniker NH June 1999

Regulation of sialomucin complex in normal rat mammary gland
Oral presentation at Nature BioTechnology Winter Symposium, Miami FL, February, 2000

Rat Muc4 (Sialomucin complex) reduces binding of anti-ErbB2 antibodies to tumor cells surfaces, a potential mechanism for Herceptin resistance
Price-Schiavi, S. A., Jepson, S., Li, P., Carvajal, M. E., Komatsu, M., and Carraway, K. L.
Poster presentation at Gordon Conference on Mammary Gland Biology, Italy, 2000

Interferon gamma (IFN-γ) blocks downregulation of sialomucin complex (SMC/Rat Muc4) expression in normal rat mammary epithelial cells
Price-Schiavi, S.A., Zhu, X., Falkenburg, R.V., Ramsauer, V., and Carraway, K. L.
Poster presentation at AACR meeting, New Orleans, LA, 2001

Personnel receiving pay from the research effort:

Shari A. Price-Schiavi
Nebila Idris

Table I- IMMUNOCYTOCHEMICAL ANALYSES OF BREAST PATHOLOGY SAMPLES

Solid nonmalignant breast and tumors (Liverpool)		**Effusions from breast cancer patients (Miami Beach)**		
Sample	**MUC4 positive**	**Sample**	**ErbB2 positive**	**MUC4 positive**
Ductal carcinoma	5/12	Pleural	8/8	5/8
Mucoid carcinoma	1/1	Ascites	5/5	4/5
Invasive lobular carcinoma	0/1			
Unknown histology	2/8			

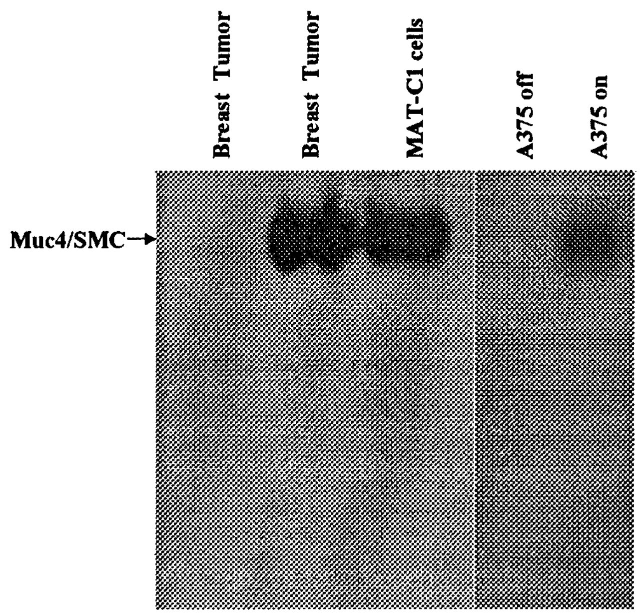
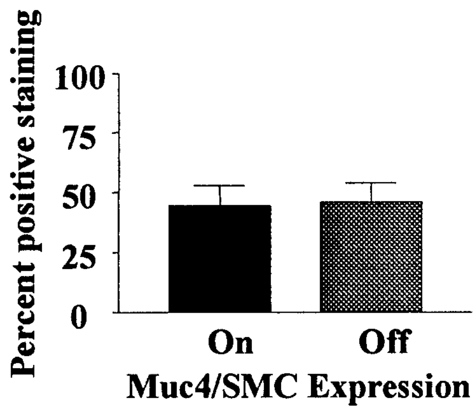


Figure 1- Immunoblot demonstration of the expression of MUC4 in human breast tumors. Infiltrating breast carcinoma specimens along with samples of A375 melanoma cells with Muc4/SMC turned ON and OFF and 13762 ascites cells were subjected to immunoblot analysis with anti-ASGP-1 mAb 15H10. Both positively and negatively staining breast tumors are shown.

A



B

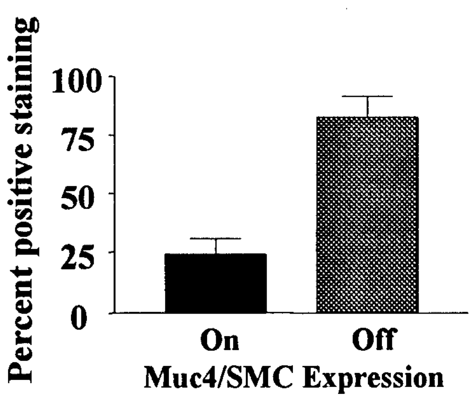


Figure 2- Effect of Muc4/SMC expression on cell surface binding of antibodies of different isotypes. A375 cells expressing high or low levels of Muc4/SMC were stained with anti-Fas IgG or anti-Fas IgM antibodies and analyzed by flow cytometry. A) A375 cells stained with anti-Fas IgG antibodies. B) A375 cells stained with anti-Fas IgM antibodies. These data are representative of three experiments.

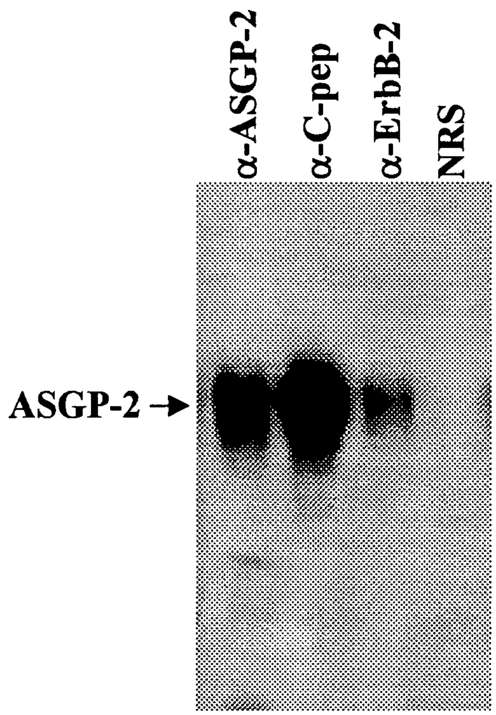


Figure 3- Co-immunoprecipitation of Muc4/SMC and ErbB-2 from A375 cells. A375 cells expressing Muc4/SMC were solubilized in RIPA buffer and cleared lysates were immunoprecipitated with anti-ASGP-2, anti-C-pep, anti-ErbB-2, or non-immune rabbit serum as indicated at the top of the figure. Immunoprecipitates were subjected to immunoblot analysis with mAb 4F12.

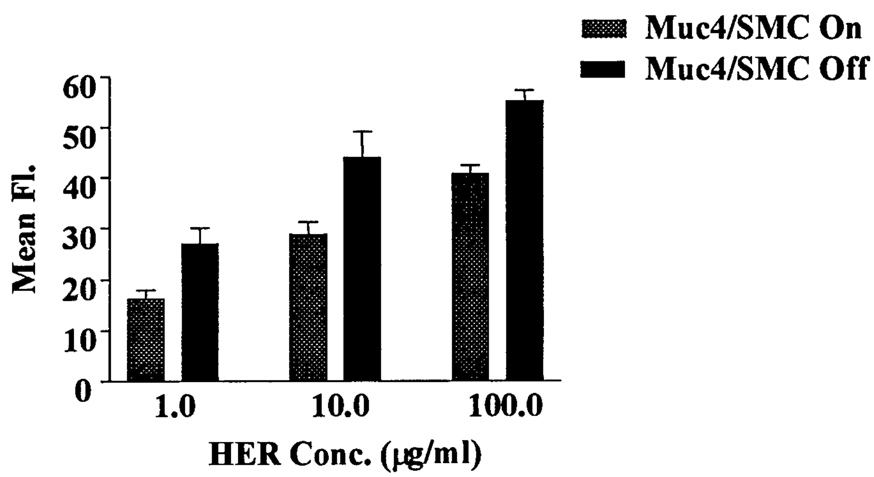


Figure 4- Effect of Muc4/SMC expression on anti-ErbB2 antibody binding in MCF-7 human breast cancer cells. MCF-7 cells stably transfected with Muc4/SMC under control of a tetracycline regulatable promoter were cultured in the presence or absence of tetracycline for 72 hours. MCF-7 cells were harvested in enzyme-free cell dissociation buffer and analyzed by FACS using Herceptin (HER) at concentrations of 100 μg/ml, 10 μg/ml, or 1 μg/ml and analyzed by flow cytometry. These data are representative of three experiments.

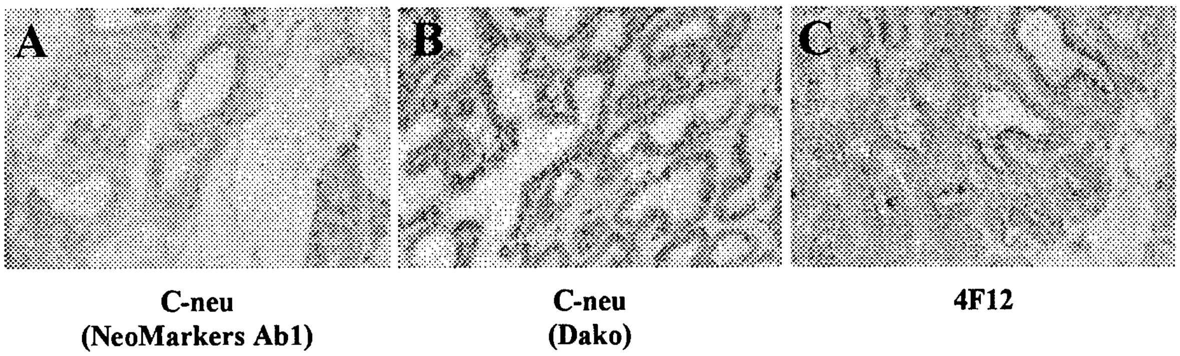
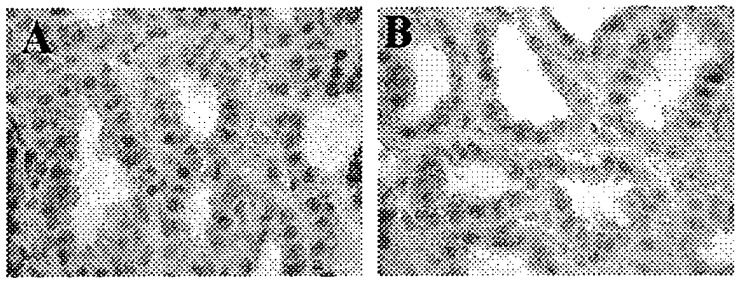


Figure 5 -Localization of Muc4/SMC and ErbB2 in lactating mammary gland. Sections (5 μm) of lactating rat mammary gland were stained with NeoMarkers anti-c-neu antibody 1 (A), Dako anti-ErbB2 (B), or anti-ASGP-2 mAb 4F12 (C) as indicated below the figure. The specifity of these antibodies and the staining controls were demonstrated in a previous study (Idris, 2001).



NeoMarkers 17 NeoMarkers 18

Figure 6 -Localization of phosphorylated ErbB2 in lactating mammary gland. Sections (5 µm) of lactating rat mammary gland were stained with NeoMarkers anti-ErbB2 antibody 17 (A) which recognizes an unphosphorylated peptide in the C-terminal region of ErbB2 or NeoMarkers anti-ErbB2 antibody 18 (B) which recognizes the phosphorylated form of the same peptide as indicated below the figure. The specifity of these antibodies and the staining controls were demonstrated in a previous study (Idris, 2001).